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(54) Title: HIGH EFFICIENCY TRANSLATION OF POLYCISTRONIC MESSAGES IN EUCARYOTIC CELLS

(57) Abstract

Methods for enhancing the expression of proteins in cultured cells derived from a multicellular organism are disclosed. The methods include the introduction into the host cell of a polycistronic transcription unit of the formula: $P\text{-}C_1\text{-}(HEL\text{-}C_n)_m$. The polycistronic transcription unit may further include a leader positioned downstream of the promoter and upstream of C_1.

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Description

HIGH EFFICIENCY TRANSLATION OF POLYCISTRONIC MESSAGES IN EUCARYOTIC CELLS

Technical Field

The present invention relates to the expression of proteins in general and, more specifically, to the high efficiency translation of polycistronic messages in cultured cells derived from multicellular organisms.

Background of the Invention

Advances in cell culture and recombinant DNA technologies have facilitated the expression of a variety of proteins of therapeutic or other economic value using genetically engineered cells. The expression of many biologically active therapeutic proteins, which are derived from higher eucaryotic sources, often requires specific post-translational modifications which do not naturally occur in lower eucaryotic or prokaryotic cells, thus necessitating the use of cells derived from higher eucaryotic sources. For example, the expression of glycoproteins in mammalian cells has the advantage of providing proteins which contain natural glycosylation. Mammalian-produced glycoproteins contain outer chain carbohydrate moieties which are markedly different from the outer chain carbohydrate moieties present on glycoproteins produced from lower eucaryotes. The use of mammalian cells as hosts for the production of secreted proteins has the significant advantage over secretion from lower eucaryotes in that mammalian cells have a secretory system that readily recognizes and properly processes secretion-directed proteins, which is not necessarily true for lower eucaryotes.

Methods for expression of cloned DNA sequences in a variety of higher eucaryotic cell lines are known in the art. Cloned DNA sequences may be introduced into mammalian cells using procedures widely reported in the literature (for review see Thilly, ed., Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA). Cell lines derived from other organisms, including avian (Kretsovali et al., Gene 58:167-176, 1987), insect (Miyajima et al., Gene 58:273-282, 1987) and fish (Isa and Shima, J. Cell. Sci 88:219-224, 1987) cell lines may also be used. In mammalian cells, the expression of cloned DNA sequences has been increased by inserting coding sequences for proteins of interest into expression units containing transcription control sequences, which include promoter sequences, enhancer sequences, leader sequences, splice signals and polyadenylation signals. Identification of clones containing transfected DNA sequences is facilitated by co-introducing a selectable marker with an expression unit. Expression levels may be optimized, for example, through amplification using selection for an amplifiable selectable marker. However, co-amplification of the expression unit is not guaranteed in every clone, particularly when the selectable marker is introduced as an independent DNA sequence.

The insertion of cloned DNA sequences into an expression unit does not guarantee efficient gene expression when the expression unit is introduced into the host cell. Low expression levels of cloned coding sequences may result from inefficient transcription or translation of the coding sequence, unstable messenger RNA (mRNA) sequences, instability of the protein production, or the presence of toxic sequences in an expression vector. The recombinant protein may be improperly, inadequately or inefficiently post-transcriptionally processed by the host cell.

Efficient expression of coding sequences in eucaryotic hosts may also require the expression of associated proteins which are required for the processing, stabilization or modification of the protein to achieve biological activity. Optimal expression of biologically active recombinant proteins may also be dependant upon the presence of translation and/or transcription factors. These proteins may be present in a host cell at such low levels that efficient expression of recombinant proteins is limited. Examples of proteins that require specific post-translational modification include certain coagulation factors, which require gamma-carboxylation of specific glutamic acid residues for biological activity and may also require the conversion of specific aspartic acid residues to beta-hydroxy aspartic acid for biological activity.

There are also certain proteins which are present as multimers in their active forms, some of which are composed of disparate subunits. Certain multimeric proteins, such as insulin, are encoded within the same cistron and are post-translationally processed into multimers containing heterologous polypeptides. Other multimeric proteins are encoded by DNA sequences which are not located within the same cistron. Examples of proteins of this type include coagulation factor XIII, which is a tetramer made of a and b chains, PDGF, which is present as an A-B dimer, immunoglobulins, hemoglobin and the major histocompatibility antigens. The expression of multimeric proteins not encoded within the same cistron may require the co-expression of all the subunits within the same cell for secretion, as has been reported for immunoglobulins (Hickman and Kornfield, J. Immunol. 121:990-996, 1978; Kearney et al, in Monoclonal Antibodies and T-Cell Hybridomas; Hammerling, Hammerling and Kearney eds., Elsevier/Northland Biomedical Press, pp 379-387, 1981;

Hendershot et al., J. Cell Biol. 104:761-767, 1987), or to achieve correct aggregation of the subunits into proper conformation to insure biological activity.

To offset the problems inherent to cloned coding sequence expression it is often advantageous to introduce into a cell other DNA sequences which function to enhance or enable expression of cloned DNA sequences. These other DNA sequences include coding sequences for processing enzymes, transcription factors, translation factors, stabilizing proteins and protease inhibitors. Optimal expression of a recombinant protein in a host cell may require the co-introduction of many coding sequences.

Methods for introducing multiple expression units into host cells include co-transfection with multiple expression vectors (Dubois et al., Proc. Natl Acad. Sci. USA 77:4549-4553, 1980; Subramani and Berg, Cell 16:777-785, 1979), transfection with vectors containing more than one expression unit (Stafford and Queen, Nature 306:77-79, 1980; Ochi et al., Proc. Natl. Acad. Sci. USA 80:6351-6355, 1983; Kadesch and Berg, Mol. Cell. Biol. 6:2593-2601, 1986) and transfection with vectors containing polycistronic transcription units (Peabody and Berg, Mol. Cell. Biol. 6:2695-2703, 1986; Kaufman et al., EMBO J. 6:187-197, 1987; Boel et al., FEBS Lett. 219: 181-188, 1987; Levinson and Simonsen, U.S. Patent No. 4,713,339). However, in practice, these methods have been shown to have severe limitations.

A major restriction for co-transfection of multiple expression vectors is the limited number of selectable markers generally in use. These selectable markers are divided into dominant markers and nondominant markers (those which provide compensating activities to cell lines which are deficient in activities complemented by the selectable marker). The

choice of an optimal selectable marker system is further limited by the few selectable markers which have been shown to co-amplify associated DNA sequences. Selectable markers are reviewed by Thilly (ibid.).

The introduction of multiple expression vectors is also limited by the number of useful cell lines which are multiply deficient in activities complemented by selectable markers or are multiply sensitive to compounds for which selectable markers provide resistance and which are also known to provide the post-translational processing required by many recombinant proteins. The identification of a cell line which appears to be a suitable host for particular selection and expression systems does not guarantee that the cell line will be amenable to selection for or amplification of the marker. The inability to perform genetic manipulation with mammalian cell lines, as is possible in lower eucaryotes and prokaryotes, requires extensive screening to identify cell lines with multiple marker deficiencies or sensitivities. Thus, the number of useful selection systems is limited with regard to transfecting cells with multiple expression units.

The probability of co-introduction of multiple expression vectors into a host cell decreases with an increase in the number of DNA sequences one seeks to co-introduce. Co-amplification of the expression units in the selected clones is also an unpredictable event. The probability of co-amplification of all co-transfected DNA sequences to an equal amplified gene dosage is reduced with the number of DNA sequences involved. Extensive and costly screening procedures will be required to identify co-amplified, co-transfected clones which contain all the transfected expression vectors in approximately the same gene dose.

As noted above, expression vectors containing more than one expression unit have been reported in the

literature (Stafford and Queen, ibid.; Ochi et al., ibid.; Lau and Kan, ibid.; Kadesch and Berg, ibid.). However, these constructs carry only two or three expression units with one expression unit on the vector containing the selectable marker. The construction of vectors containing more than three expression units is theoretically possible but such vectors have not been reported in the literature. Practically, the construction of such vectors is complicated and time consuming. The results of Kadesch and Berg (ibid.), which suggest that optimal expression is dependant on the orientation of the expression units present on the vector, further complicate the construction of vectors containing multiple transcription units by requiring orientation restrictions to avoid transcription interference. Transfection using a vector of this kind may result in recombination or rearrangement events, which may result in the shut off of certain expression units present on the vector. The identification of clones in which all the expression units are active would require extensive and costly screening.

Recent reports of genetically engineered polycistronic transcription units has raised the possibility of the expression of multiple coding sequences (cistrons) from a single promoter. However, expression of downstream cistrons in these genetically engineered polycistrons has met with only limited success. Translation from plasmid-borne polycistronic transcription units has been demonstrated (Peabody and Berg, ibid.; Kaufman et al., ibid.; Boel et al., ibid.; Levinson and Simonsen, U.S. Patent No. 4,713,339), but the level of translation of downstream cistrons from the polycistronic mRNAs was shown to be dramatically reduced.

The severe depression in downstream cistron expression demonstrated in currently reported

polycistronic transcription units renders this system unworkable for high level expression of multiple proteins in eucaryotic cells. Co-transfection with multiple expression vectors for the expression of multiple proteins is not feasible due to the limited number of available selection systems and accompanying cell lines. Expression of multiple proteins from a single vector is unwieldy and difficult due to the constraints of constructing such a vector.

There is therefore a need in the art for a means of increasing the expression of multiple proteins in higher eucaryotic cells.

Disclosure of the Invention

Briefly stated, the present invention discloses a method for enhancing the expression of proteins in cultured cells derived from a multicellular organism. The method generally comprises: (a) introducing into the cultured host cell a polycistronic transcription unit of the formula:

$P\text{-}C_1\text{-}(HEL\text{-}C_n)_m$, wherein

P is a transcriptional promoter,

C is a DNA sequence encoding a protein,

HEL is a high efficiency leader,

n is a positive integer greater than zero,

m is an integer from 1 to 8, inclusive; and

(b) growing the cultured host cell in an appropriate medium. Within a preferred embodiment, the cultured host cell is a mammalian host cell. The polycistronic transcription unit may further include a leader positioned downstream of the promoter and upstream of C_1. Preferred are viral leaders, particularly high efficiency viral leaders. Within one aspect of the present invention, C_1 and C_n may be subunits of a multi-subunit protein, such as factor XIII, platelet derived

growth factor, immunoglobulins or histocompatibility antigens.

Polycistronic transcription units for use within the method described above as well as cultured cells derived from a multicellular organism into which such a polycistronic transcription unit has been introduced are also disclosed.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 illustrates the construction of pBoel360, a plasmid containing a polycistronic transcription unit in the vector pML-1. Symbols used are Ad5 ori, the 0-1 map units of adenovirus 5; E, SV40 enhancer sequence; Ad2 MLP, the major late promoter from adenovirus 2; L1-3, the adenovirus 2 tripartite leader sequence; 5'ss, 5' splice signal; 3'ss, 3' splice signal; and pA, the late polyadenylation signal from SV40.

Figure 2 illustrates the construction of plasmid pTP/Cla.

Figure 3 illustrates the production of dicistronic mRNA. a, S1 analysis of mRNA produced by BHK cells transfected with plasmid pD5CAT-DHFR^r (arrows). Numbers on the right refer to size markers. b, diagrams of the dicistronic expression unit, dicistronic mRNA, and probe. The dotted line indicates mRNA spliced out in some transcripts.

Figure 4 illustrates the construction of plasmid pTP/F9/Cla.

Figure 5 illustrates the construction of plasmid pFVII-P-BiP.

Best Mode for Carrying Out the Invention

Prior to setting forth the invention in more detail, it may be helpful to an understanding thereof to define certain terms to be used hereinafter.

Cistron: A DNA sequence encoding a protein or polypeptide. This DNA sequence may be in the form of a gene, cDNA, or synthetic DNA fragment, or a clone thereof.

Polycistron: A DNA sequence containing at least two cistrons in which the cistrons are separated by at least the termination codon of the upstream cistron and the translation start codon of the downstream cistron. A polycistron does not contain a functional transcriptional promoter between the cistrons.

Intercistronic region: The DNA sequence between the translation termination codon of an upstream cistron and the translation initiation codon of a downstream cistron in a polycistron.

Polycistronic transcription unit: A DNA sequence containing a polycistron operably linked to a transcriptional promoter. Transcription of the polycistron results in a single mRNA containing sequences corresponding to the component cistrons.

Leader sequence: A 5′ untranslated sequence which effects efficient translation of transcribed messages.

Splice signal: A sequence showing consensus with 5′ or 3′ sequences as reported by Mount (Nuc. Acids Res. 10:459-472, 1982) that has been functionally shown to participate in a mRNA splicing event (i.e., scission and ligation reactions resulting in the excision of intervening sequences)

As briefly described above, the present invention discloses novel DNA constructs useful for enhancing the level of expression of proteins from

downstream cistrons in polycistronic transcription units. These novel DNA constructs contain polycistrons in which DNA sequences encoding proteins of interest are joined in tandem, being separated by a DNA sequence encoding a leader sequence. These polycistrons are joined to potent transcriptional and translational signals in suitable expression vectors and are introduced into cultured cells derived from multicellular organisms. Surprisingly, these constructs have been found to produce an increase in expression of the downstream cistron.

Polycistrons of the present invention may be generated by joining coding sequences with a leader sequence such that the leader sequence is between the coding sequences. Each coding sequence will have associated with it translational start and stop signals in correct reading frame. In a preferred embodiment the leader sequence is inserted into the intercistronic region using restriction endonuclease digestion and ligation. Preferred leader sequences are viral leader sequences, which include the adenovirus first leader and the adenovirus L1-IX leader (Berkner and Sharp, Nuc. Acids Res. 13:841-857, 1985), the SV40 leader and the parvovirus leader. A particularly preferred leader sequence is the high efficiency viral leader, adenovirus tripartite leader (L1-3). Suitable cellular leaders include the ovalbumin leader. It may be advantageous to append the leader sequence directly to the translation initiation sequence. In one embodiment of the present invention, the leader sequence becomes appended to the translation initiation codon following a splicing event from splice signals within the transcription unit. Within another embodiment, the leader may be joined to the translation initiation sequence by in vitro mutagenesis.

Cultured cells derived from multicellular organisms, such as mammalian cells, infected with a recombinant virus containing this potent translation signal in the intercistronic region of a polycistronic transcription unit have been found to express the downstream cistron at levels comparable to those obtained using monocistronic expression units. In the absence of the intercistronic leader, the downstream cistron is not detectably expressed in infected cells. Cells transfected with plasmids carrying polycistronic expression units including an intercistronic leader exhibit at least a 20-fold increase in protein expression from the second cistron when compared to cells containing a comparable plasmid lacking the intercistronic signal.

Appropriate leader sequences may be identified by the insertion of 5′ noncoding sequences into a viral or a plasmid test system. Such systems have been utilized to study adenovirus leader sequences (see, for example, Berkner and Sharp, Nuc. Acids Res. 13:841-857, 1985 and Kaufman, Proc. Natl. Acad. Sci. USA 82:689-693, 1985). Briefly, a potential leader sequence (a 5′ noncoding sequence) of interest is inserted into an expression unit comprising at least a transcriptional promoter operatively linked to a marker coding sequence, such as DHFR, such that the potential leader sequence is inserted immediately 5′ to the translation initiation site of the marker coding sequence. The marker coding sequence is preferably one for which an assay exists. Marker coding sequences include DHFR and hepatitis B surface antigen (Davis et al., Proc. Natl. Acad. Sci. USA 82:7560-7564, 1985).

To test potential leader sequences in a plasmid system, expression units containing the leader sequence of interest are inserted into a vector capable of transfecting higher eucaryotic cells. Vectors

suitable for transfecting mammalian cells include derivatives of pBR322 (Bolivar et al., Gene 2:95-113, 1977), such as pML-1 (Lusky and Botchan, Nature 293:79-81, 1981), and derivatives of the pUC (Messing, Meth. Enzymol. 101:20-79, 1983) vectors. Vectors suitable for use in transfecting other host cells are described by, for example, Kretsovali et al. (ibid.), Miyajima et al. (ibid.) and Isa and Shima (ibid.). The resultant expression vectors are transfected into host cells and the expression of the marker is compared to the marker expressed by cells transfected with the expression vector without the leader sequence. At least a five-fold increase in marker expression in cells transfected with an expression vector containing a leader sequence over cells transfected with an expression vector without a leader sequence identifies a suitable leader sequence. A greater than five-fold increase in expression with an expression vector carrying the leader sequence identifies a high efficiency leader. Leader sequences may be tested in a viral system, such as adenovirus, by constructing recombinant viruses with the plasmid expression units described above. The resultant recombinant viruses are used to infect host cells and the levels of marker expression are measured. At least a five-fold increase in marker expression in cells infected with an expression unit containing a leader sequence over cells infected with an expression unit without a leader sequence identifies a suitable leader sequence. A greater than five-fold increase in marker expression with an expression unit carrying a leader sequence identifies a high efficiency leader sequence. After suitable leaders are identified, they are used in constructing polycistronic expression vectors as described below. Such vectors will contain additional genetic elements which facilitate the high level expression of the protein(s) of interest.

For production of a protein of interest, the polycistronic expression units of the present invention are inserted into vectors. Suitable vectors include recombinant plasmids as described above. Recombinant virus vectors include the SV40 viral vectors and adenovirus vectors (for review see, Thilly, ibid.). A particularly preferred vector is a recombinant adenovirus (hereinafter "Ad") vector. Expression of cDNAs using Ad vectors has been achieved, for example by Berkner et al. (Nuc. Acids. Res. 13:841-857, 1985). Recombinant adenovirus vectors containing polycistronic transcription units provide the advantage of introducing the transcription unit into virtually every cell of any tissue or cell line. This may be particularly advantageous if the polycistronic transcription unit is to be used in gene therapy.

In order to obtain expression of the polycistronic transcription units containing one or more DNA sequences encoding proteins of interest, the vector will normally contain additional elements. A transcriptional promoter is positioned upstream of the translation initiation signal of the first cistron. Suitable promoters include the mouse metallothionein (MT-1) promoter (Palmiter et al., Science 222:809-814, 1983), the SV40 late promoter (Piatak et al., J. Virol 48:503-520, 1983), the SV40 early promoter (Benoist and Chambon, Nature 290:304-310, 1981), and the cytomegalovirus (CMV) promoter. Viral promoters are preferred due to their efficiency in directing transcription. A particularly preferred promoter is the major late promoter from adenovirus, although any efficient promoter can be utilized in the disclosed methodology. It may be advantageous to include, in an expression unit, a leader sequence located downstream of the promoter and upstream of the first cistron. Preferred leader sequences include the adenovirus first

leader, the adenovirus L1-IX leader and the ovalbumin leader. A particularly preferred leader sequence is the adenovirus tripartite leader. Suitable leader sequences may be identified by screening methods described above. The expression unit may contain splice signals composed of a 5′ splice signal and a 3′ splice signal. The 5′ splice signal may be associated with a leader sequence such as any of those mentioned above. In a preferred embodiment the 5′ splice signal is a sequence associated with the adenovirus L3 leader. The 3′ splice signal may be any one of a number of splice signals (for review see Mount, ibid.) such as the rabbit beta-globin 3′ splice signal (Ruskin et al., Cell 38:317-331, 1984). A particularly preferred 3′ splice signal is from the variable region of an immunoglobulin gene. Also contained in the expression unit is a polyadenylation signal located downstream from the DNA sequences comprising the polycistron. Viral polyadenylation signals, such as the early or late polyadenylation signals from SV40 or the polyadenylation signal from the adenovirus E1b region, are particularly preferred. Polyadenylation signals may also be supplied by the coding sequences present in the polycistron. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer, preferably located upstream of the promoter.

In some instances, it is preferred that a selectable marker be introduced into the cells along with the polycistronic transcription unit. Selectable markers include the neomycin resistance gene, the hygromycin gene, the Ecogpt gene, the thymidine kinase gene, the adenine phophoribosyltransferase gene, the hypoxanthine phosphoribosyltransferase gene, and multiple drug resistance factors (Roninson et al., Proc. Natl. Acad. Sci USA 83:4538-4542, 1986; Ueda et al., J. Biol. Chem. 262:505-508, 1987). Preferably, the

selectable marker will be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene. A particularly preferred amplifiable selectable marker is the $DHFR^r$ cDNA (Simonsen and Levinson, Proc. Natl. Acad. Sci. USA 80:2495-2499, 1983). Selectable markers are reviewed by Thilly (ibid.) and the choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell as separate DNA sequences at the same time as the polycistronic transcription unit, as cistrons in the polycistronic trancription unit, or as independent expression units on the same vector. It may be particularly advantageous to place an amplifiable selectable marker as the terminal cistron in a polycistronic transcription unit such that the penultimate cistron and the selectable marker are not separated by a leader sequence. A selectable marker so placed is translated at a reduced efficiency and forces the amplification of the selectable marker and its associated DNA sequences to compensate for the selective conditions.

Polycistronic transcription units as described within the present invention have wide application in the production of recombinant proteins in eucaryotic cells. These applications include the use of polycistronic messages to produce commercial quantities of therapeutic and commercially important proteins or to be used in applications of gene therapy. Polycistronic messages may be adapted to encode proteins of interest and coding sequences which enhance or enable the expression of biologically active proteins of interest. Proteins which function to enhance or enable the expression of recombinant proteins include processing enzymes, protease inhibitors, stabilizing factors,

transcription factors, translation factors and selectable markers.

As noted above, polycistronic transcription units may be utilized in combination with an adenovirus vector system in gene therapy. Gene therapy, as used herein, is the insertion into an organism of a DNA coding sequence which corrects a genetic defect in the host organism. At present, the only human tissues which have been used for gene transfer are bone marrow and skin cells. Polycistronic transcription units may be constructed which allow efficient expression of proteins of interest in tissues where those proteins do not occur in nature. Current studies on human gene therapy have concentrated on using human genes introduced into human cells by insertion into retroviruses. Because there have been no reported rearrangement of intron-containing genes or inhibition due to promoter or polyadenylation signals in adenovirus vectors, as observed with retrovirus vectors, adenovirus vectors may prove to be advantageous over retrovirus vectors for the introduction of genes for gene therapy. Adenovirus is able to transform a variety of human and rodent cell lines to generate stable integrants and it has a proven ability to penetrate every cell during infection. Thus, a highly infective, transforming, recombinant adenovirus vector may prove to be advantageous over retrovirus vectors for gene therapy.

DNA sequences encoding therapeutic and economically useful proteins which may be expressed in polycistronic transcription units include, but not are restricted to, those coding for blood coagulation factors, a variety of serine proteases, growth factors, protein C, protein S, tissue plasminogen activator, immunoglobulins, histocompatibility antigens, plasminogen, anti-inflammatory proteins, anticoagulants and analogs and derivatives of these proteins.

Many of these proteins occur as multi-subunit proteins whose coding sequences are not located within the same cistron. Expression of multi-subunit proteins in mammalian cells requires the expression of coding sequences for all of the subunits. Multi-subunit proteins include factor XIII, platelet-derived growth factor, immunoglobulins, the major histocompatability antigens and hemoglobin. Examples of multi-subunit proteins which may require co-expression of component subunits within the same host cell are factor XIII and immunoglobulins. Factor XIII is a tetramer composed of a_2 and b_2 dimers (Chung et al., J. Biol. Chem. 249:940-950, 1974). It has been shown that the b_2 dimer acts to stabilize the a_2 dimer. Immunoglobulins are known to require expression of both heavy and light chains for secretion of functional immunoglobulins (Hickman and Kornfield, ibid; Kearney et al., ibid.; Hendershot et al., ibid.).

Polycistronic messages may also encode processing proteins which are required in enhanced levels to efficiently produce high levels of biologically active proteins. Processing proteins include proteases which cleave a precursor protein at a particular site to provide the mature and/or active form of a protein or proprotein, or which cleave a single-chain protein to a multi-chain form. Other examples of processing proteins are those which modify amino acids, such as gamma-carboxylase, an enzyme which modifies specific glutamic acid residues of certain coagulation factors and other calcium binding proteins; enzymes responsible for the conversion of aspartic acid to beta-hydroxy aspartic acid, a modification necessary for the biological activity of protein C; and enzymes responsible for hydroxylation of proline residues. Other processing proteins include enzymes responsible for myristoylation, C-terminal amino acid removal,

sulfation, C-terminal amidation and the addition of carbohydrate chains to glycoproteins. One example of a processing enzyme which is not naturally found in mammalian cells and may be incorporated into a polycistronic transcription unit is the S. cerevisiae KEX2 gene product. The KEX2 gene product is an endopeptidase which cleaves at Lys-Arg residues. By way of example, a KEX2 coding sequence and a protein C coding sequence are encoded in a polycistronic transcription unit. The KEX2 gene product facilitates processing of the precursor form of protein C. It may be preferable to place the sequence encoding the secreted protein, protein C, in the first cistron position to facilitate secretion.

Polycistronic transcription units may also include coding sequences for stabilizing proteins. Stabilizing proteins include protease inhibitors which block the proteolytic degradation of the protein of interest; proteins which bind to the protein of interest making it unavailable to degrading enzymes; proteins which bind to proteins as co-factors, or other molecules required by a protein; and proteins which inactivate co-factors. An example of a coding sequence which functions to stabilize or facilitate activation of another protein in a polycistron is protein S. Protein S functions to bind to protein C and allows acceleration in the activation of protein C. Thus, a protein C-protein S polycistron may improve expression of activated protein C. An example of a stabilizing protein is von Willebrand factor (vWF). A polycistron which encodes both vWF and coagulation factor VIII will produce vWF, which functions to stabilize coagulation factor VIII and may increase the half life of the factor VIII in the extracellular medium.

Polycistronic transcription units may incorporate DNA sequences which encode transcription or

translation factors (Dynan and Tijan, Nature 316:774-777, 1985; Baeuerle and Baltimore, Cell 53:211-217, 1988; Hattman et al., Gene 55:345-351, 1987; Kaufman et al., Mol. Cell. Biol. 7:3759-3766, 1987). Incorporation of these factors may effect better expression by supplementing endogenous cellular machinery thereby providing more efficient gene expression. An example of a cloned transcription factor is the promoter-specific sp1 (Dynan and Tijan, ibid.). A polycistronic transcription unit comprising the coding region for sp1 linked to a gene or cDNA of interest which encodes or has been modified to encode sp1 recognition sequences may allow greater transcription of the coding sequence of interest.

It may be advantageous to include sequences in polycistronic transcription units that encode proteins which facilitate the secretion of other proteins. An example of a polycistronic transcription unit which encodes a secreted protein and a protein which may aid in the secretion of other proteins is one which contains factor VII and BiP coding sequences. The immunoglobulin binding protein BiP is found in the lumen of the endoplasmic reticulum (ER) and has a C-terminal amino acid sequence of Lys-Asp-Glu-Leu (Munro and Pelham, Cell 46:291-300, 1986). Munro and Pelham (ibid.) note that the KDEL tetrapeptide may be part of a signal which causes retention of the proteins in the ER. The secretion of factor VII, which shares some homology with the C-terminal amino acid sequence of BiP, may be retarded due to the presence of a potential retention signal. The co-expression of BiP, which is preferentially retained within the ER, with factor VII, may saturate the host ER retention system, thus allowing factor VII to be more efficiently secreted.

Expression vectors according to the present invention may be introduced into cultured cells by, for

example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:724, 1975, Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virol. 52:456-467, 1973) or by electroporation (Neumann, EMBO J. 1:841-845, 1982). Viral expression vectors may also be used to infect a host cell using methods described by, for example, Kaufman (Proc. Natl. Acad. Sci. USA 82:689-693, 1985) It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture which is introduced into the cells.

A variety of higher eucaryotic host cells or cultured cells derived from a multicellular organism may be used within the present invention. Cells which can be grown in culture and express cloned DNA sequences include cells of mammals, insects (Miyajima et al., ibid.), amphibians (Wolf and Quimby, Science 144:1578, 1964 and Freed et al., Biology of Amphibian Tumors, Mizell ed., pp 101-111, Springer Verlag, 1969), reptiles (Clark, J. Natl. Cancer Inst. 43:1097-1102, 1969; Clark, J. Natl. Cancer Inst. 46:309-321, 1971), and birds (Prier, J. Virol. 2:178, 1968 and Kretsovali et al., Gene 58:167-176, 1987). Preferred mammalian cell lines for use in the present invention include the COS (ATCC CRL 1650), BHK (ATCC CCL 10), CHO and 293 (ATCC 1573) cell lines as well as derivatives and isolates of these cell lines, although it will be evident to those skilled in the art that other cell lines may be preferred for production of particular proteins. Mammalian tissue may also be suitable for use in the present invention. In general, a host cell line or tissue will be selected on the basis of its ability to produce the protein of interest at a high level and/or its suitability for use with a desirable selectable marker. However, the present invention allows one to produce virtually any protein in practically any cell line which can be grown in vitro.

After the expression vectors have been introduced into the host cells, the cells are generally allowed to grow for a period of time, typically 1-2 days, to begin expressing the gene of interest. Cells containing expression vectors according to the present invention may be grown in an appropriate medium. Medium for facilitating growth of cultured eucaryotic cells contains a carbon source, amino acids and vitamins in a balanced salt solution supplemented with defined growth factors or serum. The selection of an appropriate medium for optimizing cell growth is well within the level of ordinary skill in the art and is dependent upon the specific characteristics of the tissue or cell line being grown. Media formulations are well known in the art (see, for example, Mammalian Cell Culture: The Use of Serum-Free Hormone-Supplemented Media, Mather ed., Plenum Press, New York, NY, 1984; Thilly, ibid.; and the catalogs of the American Type Culture Collection, Rockville, Md.) and may be made according to published formulations or may be obtained from commercial sources (for example, Gibco-Life Technologies Inc., Lawrence, MA; American Type Culture Collection, Rockville, Md.). Selection pressure is applied to select for the growth of cells which are expressing the selectable marker. When using methotrexate selection, for example, increasing the concentration of the drug in a stepwise manner allows selection for increased copy number of the cloned sequences, resulting in increased expression levels. Clones of such cells may be screened for production of the protein of interest. Cells are grown and proteins are isolated from the cells by lysis. Useful screening methods include immunological assays and activity assays.

Methods for purification of recombinant proteins are generally known in the art. Where the protein is retained within the host cell, it will be

necessary to first disrupt the cell and remove cell debris, preferably by centrifugation, to produce a cleared lysate. In the case of a secreted protein, the protein is purified directly from the culture medium. The cleared lysate or medium is fractionated by conventional protein purification methods. A multi-step process will generally be used. Typical procedures in this regard include precipitation (e.g., with polyethylene glycol or ammonium sulfate), ion exchange chromatography, affinity chromatography, preparative gel electrophoresis, high performance liquid chromatography, and fast pressure liquid chromatography. In some cases it is preferable to concentrate the fractions of interest between steps, such as by ammonium sulfate precipitation followed by dialysis to remove excess salt. The selection and ordering of the various steps will depend on the characteristics of the particular protein of interest, and is within the level of ordinary skill in the art.

The following examples are offered by way of illustration, and not by way of limitation.

Example 1: Construction of pD3

Plasmid pD3, comprising the Ad5 ori, SV40 enhancer, Ad2 major late promoter, Ad2 tripartite leader, 5′ and 3′ splice signals, a DHFR cDNA, the SV40 polyadenylation signal and pML-1 vector sequences, was constructed to introduce a unique Bcl I site 5′ to the DHFR coding sequence. To construct plasmid pD3, the Pst I site immediately downstream from the DHFR coding sequence in pDHFRIII was converted to a Bcl I site. The adhesive ends of Pst I partially digested pDHFRIII were deleted using dCTP in the presence of T_4 DNA polymerase. The DNA was then ethanol precipitated and ligated to

kinased Bcl I linkers. The resultant ligation mixture was digested with Bcl I and was electrophoresed through an agarose gel. The 5.8 kb fragment was isolated from the gel and was recircularized by self-ligation. A plasmid having the desired modification was selected and designated pDHFR'. Plasmid pDHFR' was transformed into a dAM⁻ E. coli strain and plasmid DNA was prepared.

Plasmid pD2' was then generated by cleaving pDHFR' and pSV40 (comprising Bam HI-digested SV40 DNA cloned into the Bam HI site of pML-1) with Bcl I and Bam HI to isolate the 0.2 kb SV40 polyadenylation signal and the 4.9 kb pDHFR' fragment. The 0.2 kb pSV40 fragment and the 4.9 kb pDHFR' fragment were then ligated to construct plasmid pD2'.

Plasmid pD2' was modified by deleting the "poison" sequences in the pBR322 region (Lusky and Botchan, Nature 293:79-81, 1981). Plasmids pD2'and pML-1 were digested with Eco RI and Nru I and the fragments were separated by agarose gel electrophoresis. The 1.9 kb pD2' fragment and the 1.8 kb pML-1 fragment were isolated and ligated together. A plasmid having the desired structure was selected and designated pD2. This plasmid was then digested with Eco RI and Bgl II and a 2.8 kb fragment, comprising the 3' splice signal, SV40 polyadenylation signal and pML-1 vector sequences, was isolated and designated fragment C.

To generate the remaining fragments used in constructing pD3, pDHFRIII was modified to convert the Sst II site into either a Hind III or a Kpn I site. Plasmid pDHFRIII was digested with Sst II, incubated with dCTP and T_4 DNA polymerase, and ligated to kinased Hind III or Kpn I linkers. The resultant plasmids were digested with Hind III or Kpn I, as appropriate, and electrophoresed through agarose. Gel-isolated DNA was then religated and used to transform E. coli RR1. The resultant plasmids were designated pDHFRIII(Hind III)

and pDHFRIII(Kpn I). A 700 bp Kpn I-Bgl II fragment (fragment A) was then purified from pDHFRIII(Kpn I) by digestion with Bgl II and Kpn I followed by agarose gel electrophoresis.

The SV40 enhancer sequence was inserted into pDHFRIII(Hind III) in the following manner. SV40 DNA was digested with Hind III and the Hind III C SV40 fragment (5089-968 bp) was gel purified and inserted in the Hind III site of pDHFRIII(Hind III). The resultant plasmid, pE2, was then digested with Eco RI and Kpn I, and a 700 bp fragment, containing the Ad5 ori and the SV0 enhancer (designated fragment B), was isolated.

For the final construction of pD3, fragments A, B and C were joined in a three-part ligation and the mixture was used to transform E. coli RR1. Positive colonies were selected and the vector was designated pD3.

Example 2: Construction of pD5 and pD11

Plasmids pD5 and pD11, comprising the Ad5 ori, SV40 enhancer, Ad2 major late promoter, Ad2 tripartite leader (L1-3), 5′ and 3′ splice signals, the SV40 polyadenylation signal and pML-1 vector sequences, were constructed to introduce a unique Bam HI site. To construct pD5 and pD11, pDHFRIII was first modified by converting the Pst I site immediately upstream from the DHFR sequence to a Bam HI site by incubating Pst I partially digested pDHFRIII with dCTP in the presence of T_4 DNA polymerase. The DNA was then ethanol precipitated and ligated to kinased Bam HI linkers. Excess linkers were removed by digestion with Bam HI followed by gel electrophoresis and isolation of the 4.9 kb fragment. The 4.9 kb fragment was recircularized by self-ligation. A plasmid having the desired modification was selected and designated pD1′.

Plasmid pD1 was then generated from pD1' by first cleaving pSV40 (comprising Bam HI digested with SV40 DNA cloned into the Bam HI site of pML-1) with Bcl I and Bam HI to isolate the 0.2 kb SV40 polyadenylation signal. Plasmid pD1' was linearized by digestion with Bam HI and the 4.9 kb fragment was isolated. These fragments were then ligated with the SV40 polyadenylation sequence in the late orientation to construct plasmid pD1.

Plasmid pD1 was modified by deleting the "poison" sequences in the pBR322 region (Lusky and Botchan, Nature 293:79-81, 1981). Plasmids pD1 and pML-1 (Lusky and Botchan, ibid.) were digested with Eco RI and Nru I and the fragments were separated by agarose gel electrophoresis. The 1.9 kb pD1 fragment and the 1.8 kb pML-1 fragment were isolated and ligated together. A plasmid having the desired structure was selected and designated ppD1. This plasmid was then digested with Eco RI and Bgl II and a 2.8 kb fragment, comprising the 3' splice signal, SV40 polyadenylation signal and pML-1 vector sequences, was isolated and designated fragment C'.

The SV40 enhancer sequence was inserted into pDHFRIII(Hind III) in the following manner. SV40 DNA was digested with Hind III and the Hind III C SV40 fragment (5089-968 bp) was gel purified and inserted in the Hind III site of pDHFRIII(Hind III) by ligation. The ligation mixture was transformed into E. coli RR1. Plasmid DNAs prepared from the transformants were screened by restriction enzyme analysis. Two plasmids were isolated. One, designated pE1, contained the enhancer oriented with the Kpn I site distal to the Ad5 ori and the other, designated pE2, contained the enhancer oriented with the Kpn I site proximal to the Ad5 ori. Plasmid pE1 was then digested with Eco RI and Kpn I, and a 700 bp fragment, containing the Ad5 ori and

the SV40 enhancer (designated fragment B'), was isolated. Plasmid pE2 was digested with Kpn I and Bgl II to isolate the 0.9 kb fragment (fragment D').

The remaining fragments used in constructing pD11 and pD5 were obtained from pDHFRIII (Kpn I). Plasmid pDHFRIII(Kpn I) (Example 1) was digested with Kpn I and Bgl II to isolate the 700 bp Kpn I-Bgl II fragment (designated fragment A') and EcoR1 and Kpn I to isolate the 0.4 kb fragment comprising the Ad5 ori (fragment E').

For the final construction of pD5 and pD11, fragments A', B' and C' were joined in a three-part ligation and fragments C', D' and E' were joined in a three-part ligation. The mixtures were then transformed into E. coli RR1. Plasmid DNA was prepared and analyzed by restriction enzyme analysis. A plasmid from the ligation of fragments A', B' and C' was designated pD5 (Figure 1). A plasmid from the ligation of fragments C', D' and E' was designated pD11 (Figure 4).

Example 3: Construction of pBoel360 and Ad5 (CAT-DHFR)

Plasmid pBoel360, comprising a polycistronic transcription unit containing a mouse $DHFR^r$ cDNA and a CAT gene, was constructed from the precursor plasmids pDHFRI (Berkner and Sharp, Nuc. Acids Res. 12:1925-1941, 1984), pDHFRIII (Berkner and Sharp, Nuc. Acids. Res. 13:841-857, 1985) and pD5CAT.

The $DHFR^r$ cDNA was constructed from the wild-type DHFR cDNA as described by Boel et al. (FEBS Lett. 219:181-188, 1987). Briefly, plasmid pDHFRI was digested with Bgl II and Bam HI to isolate the 1050 bp fragment comprising the DHFR cDNA. The Bgl II-Bam HI fragment was joined in a two-part ligation to pEMBL8 (Dente et al., Nuc. Acids Res. 11:1645-1655, 1983) which had been linearized by digestion with Bam HI. A plasmid

containing the insert in the proper orientation was designated pDHFR/pEMBL8. Single-stranded pDHFR/pEMBL8 DNA was prepared and was subjected to site-directed in vitro mutagenesis using the method of Zoller and Smith (DNA 3:479-488, 1984) and the mutagenic oligonucleotide ZC165 (5' GAG GCC AGG GTC GGT CTC CG 3'). Mutagenesis using ZC165 introduced a Leu to Arg mutation at position 22 of the DHFR cDNA resulting in the $DHFR^r$ mutant cDNA as described by Simonsen and Levinson (Proc. Natl. Acad. Sci. USA 80:2495-2499, 1983). Positive colonies were identified by colony hybridization with labeled ZC165 and the mutation was confirmed by dideoxy sequencing. Replicative form DNA was prepared from a positive plaque and was digested with Xho II to isolate the fragment comprising the $DHFR^r$ cDNA. Plasmid pDHFRI was digested with Bgl II and Bam HI to isolate the vector-containing sequences. The Xho II pDHFRI fragment was joined in a two-part ligation to the $DHFR^r$ cDNA fragment. A plasmid containing the $DHFR^r$ cDNA in the proper orientation was designated $pDHFR^rI$ (Figure 1).

Plasmid pD5CAT was a progenitor plasmid for the CAT-$DHFR^r$ polycistronic transcription unit and was constructed from the progenitor plasmid pD5. A chloramphenicol transacetylase (CAT) gene was inserted into the Bam HI site of pD5 to construct plasmid pD5CAT as follows. Plasmid pD5 was linearized by digestion with Bam HI. The CAT cDNA was provided as a Bam HI-Xho II fragment and was joined with the linearized pD5 in a two-part ligation. A plasmid containing the CAT cDNA fragment in the correct orientation was designated pD5CAT (Figure 1).

A polycistronic transcription unit was constructed in pD5CAT, as shown in Figure 1. Plasmid $pDHFR^rI$ was digested with Fnu 4HI and the adhesive ends were blunted by treatment with T4 DNA polymerase and the four deoxyribonucleotide triphosphates. Kinased Bam HI

linkers were added to the blunted fragments and the linkered fragments were digested with Bam HI and Nco I. A 0.62 kb fragment, comprising a portion of the DHFR^r cDNA, was isolated by gel electrophoresis and electroelution. Plasmid pDHFR^{r}I was also digested with Xba I and partially digested with Nco I to isolated the fragment comprising the 3' end of the DHFR^r cDNA, the SV40 polyadenylation signal and 276 bp of pBR322 vector sequence. Plasmid pD5CAT was digested with Xba I and partially digested with Bam HI to remove the SV40 polyadenylation signal and 276 bp of pML vector sequences. The Xba I-Bam HI vector and CAT gene fragment of pD5CAT was joined with the Nco I-Xba I fragment and the Bam HI-Nco I fragment in a three part ligation. The resulting plasmid was designated pD5CAT-DHFR^r.

Figure 1 diagrams the modification of plasmid pD5CAT-DHFR^r to change the Bam HI site 3' to the SV40 polyadenylation signal to a Cla I site. Plasmid pD5CAT-DHFR^r was digested with Xba I and Sst I to isolate the 0.9 kb fragment comprising part of the DHFR^r cDNA, the SV40 polyadenylation signal and pML-1 vector sequences. The 0.9 kb fragment was joined to Xba I-Sst I linearized pUC13 by ligation. The resultant plasmid, designated pBoel360a, was digested with Bam HI and the adhesive ends were filled in by treatment with DNA polymerase I (Klenow fragment) and the four deoxyribonucleotide triphosphates. The DNA was recircularized by ligation and the resultant plasmid, which contained a Cla I site in place of the Bam HI site of pBoel360a, was designated pBoel360b. Plasmid pBoel360b was then digested with Xba I and Sst I and the 0.9 kb insert was isolated. Plasmid pD5CAT-DHFR^r was digested with Xba I and Sst I to remove the 0.9 kb fragment comprising the 3' portion of the DHFR^r cDNA, the SV40 polyadenylation signal and the pML vector sequences. The Xba I-Sst I vector-containing

fragment from pD5CAT-DHFR^r was joined with the 0.9 kb Xba I-Sst I fragment from pBoel360b. The resultant plasmid was designated pBoel360 (Figure 1).

A recombinant adenovirus vector was generated by cotransfecting 293 cells with pBoel360 and incomplete Ad5 viral DNA essentially as described by Stow (J. Virol. 37:171-180, 1981) and Berkner and Sharp (Nuc. Acids Res. 11:6003-6020, 1983). An Xba I site was substituted for the Bgl II site at mu 9.4 of Ad5 and the Xba I 9.4-100 mu fragment was prepared. This fragment was ligated to Xba I-digested pBoel360, and 10 mg of DNA was used to transfect the cells by the calcium phosphate method. Recombinant virus was recovered and designated Ad5(CAT-DHFR).

ΔE1?

Example 4: Construction of pGEMcGM/Eco/1100

A cDNA encoding a portion of the L1-3 leader fused to the human granulocyte-macrophage colony-stimulating factor coding sequence (hGM-CSF) was cloned from mRNA isolated from COS cells which were transiently expressing hGM-CSF from pDg GM II (Kaushansky et al., Biochemistry 26:4861-4867, 1987). Briefly, a 2.6 kb Bst EII-Eco RI genomic fragment encoding hGM-CSF was subcloned into Bst EII-Eco RI cut pD3 (Example 1). The resultant plasmid, pDgGM II, was transfected into COS cells as described by Kaushansky et al. (Proc. Natl. Acad. Sci. USA 83:3101-3105, 1986).

RNA, prepared essentially as described by Chirgwin et al. (Biochemistry 18:5294-5299, 1979), was used as a template in the preparation of a lambda gt11 cDNA library using an adaptation of the method described by Gubler and Hoffman (Gene 25:263-269, 1983).

A positive cDNA clone, identified by hybridization to labelled hGM-CSF genomic probes, was found to contain DNA sequences from the cap site through

the coding region and into the 3'-untranslated region. A complete cDNA clone comprising a portion of the L1-3 fused to the hGM-CSF coding sequence was isolated from the lambda phage by digestion with Eco RI and ligated into the unique Eco RI site of pGEM-1 (Promega Biotec, Madison, WI). The resultant plasmid, designated pGEMcGM.Eco.1100, contained two copies of the hGM-CSF cDNA oriented in tandem in the pGEM-1 vector (Figure 2).

Example 5: Construction of pTP/Cla and Ad5(TPCla)

The L1-3 leader sequence used in the intercistronic region of the polycistronic transcription unit was derived from plasmids pGEMcGM.Eco.1100 (Example 4) and plasmid D^S/PUC, comprising the Ad5 ori, Ad2 major late promoter (MLP), Ad2 L1-3 and its accompanying 5' splice signal, immunoglobulin 3' splice site, a DHFR cDNA, the SV40 polyadenylation signal and pUC13 vector sequences. As shown in Figure 2, plasmid pGEMcGM.Eco.1100 was digested with Bcl I to isolate the 0.8 kb fragment comprising the CSF cDNA fused to the L1-3 leader sequence. Oligonucleotides ZC582 (5' AAT TCC CGG G 3') and ZC583 (5' GTA CCC CGG G 3') were kinased and annealed using conditions generally described by Maniatis (ed. Molecular Cloning A Laboratory Manual, Cold Spring Harbor, NY, 1982). The kinased, annealed oligo-nucleotides formed a Bam HI-Eco RI adaptor which was joined to the 0.8 kb pGEMcGM.Eco.1100 fragment by ligation. The ligation mixture was digested with Eco RI to isolate the 0.2 kb fragment comprising the L1-3 leader sequence. Plasmid pUC13 was linearized by digestion with Eco RI and was subsequently treated with calf intestinal phosphatase to prevent recircularization. The 0.2 kb L1-3 fragment was joined to the linearized pUC13 by ligation. The resultant plasmid was designated CSF L1-3 #12 (#265) (Figure 2).

The 5′ portion of the L1-3 sequence was taken from pD^S/PUC which was constructed as follows. Plasmid pD^S/PUC is derived from plasmid pDHFRIII which has been disclosed by Berkner and Sharp (ibid., 1985). Plasmid pDHFRIII was modified to place a unique Kpn I site at the Sst II site between the Ad5 ori and the Ad2 MLP. Plasmid pDHFRIII was linearized by digestion with Sst II and the linear fragment was blunt-ended by treatment with T4 DNA polymerase and the appropriate nucleotides. The linear fragment was joined with kinased Kpn I linkers. Excess linkers were removed by digestion with Kpn I and the linear fragment was self-ligated. The resultant plasmid was designated pDHFRIII/SSt->Kpn I. Plasmid pDHFRIII/Sst->Kpn I was digested with Eco RI and Xba I to isolate the 2.4 kb fragment comprising the Ad5 ori, Ad2 MLP, 5′ splice signal, 3′ splice signal, a DHFR cDNA, SV40 polyadenylation signal and approximately 300 bp of pBR322 vector sequences. The 2.4 kb fragment was ligated with pUC13 which had been linearized by digestion with Eco RI and Xba I. The resultant plasmid was designated pD^S/PUC.

As shown in Figure 2, plasmid pD^S/PUC was digested with Eco RI and Pst I to isolate the 1.1 kb fragment comprising the Ad5 ori, Ad2 MLP and L1-3, and 5′ and 3′ splice signals. The 1.1 kb fragment was digested with Hha I to isolate the 500 bp fragment comprising the L1-3 and associated 5′ and 3′ splice signals. The 500 bp fragment was subsequently treated with T4 DNA polymerase to blunt the adhesive ends. The blunt-ended fragment was ligated to kinased Bam HI linkers. Excess linkers were removed by digestion with Bam HI and the linkered fragment was inserted into the Bam HI site of pUC13. The resultant plasmid was designated L1-3 Bam-Pst (#301) (Figure 2).

Figure 2 shows the construction of plasmid pTP(#323). Plasmid CSF L1-3 #12 (#265) was digested

with Xho I and Nde I to isolate the 2.5 kb fragment comprising the 3′ portion of the L1-3 and the pUC13s vector sequences. Plasmid L1-3 Bam-Pst (#301) was digested with Bam HI and Xho I to isolate the 0.17 kb fragment comprising the 5′ portion of the L1-3. The E. coli vector pUC13 was digested with Bam HI and Nde I to isolate the 200 bp vector fragment. The 0.17 kb L1-3 fragment from L1-3 Bam HI-Pst I (#301) was joined with the 2.5 kb fragment from CSF L1-3 #12 (#265) and the 200 bp fragment of pUC13 in a three part ligation. The resultant plasmid was designated pTP (#323) (Figure 2).

Plasmid pTP (#323) was digested with Bam HI to isolate the 0.2 kb L1-3 fragment. Plasmid pBoel360 (also known as Cla I (#221) and described in Example 2) was subjected to a partial digestion with Bam HI to linearize the plasmid. The linearized pBoel360 was joined with the 0.2 kb Bam HI fragment of pTP (#323) in a two part ligation. The ligation mixture was transformed into E. coli HB101. Plasmid DNA was isolated from the transformants and analyzed by restriction enzyme digestion to verify the correct orientation of the component fragments. A plasmid containing the L1-3 fragment inserted in the correct orientation into the intercistronic region between CAT and DHFR was designated pTP/Cla (#431) (Figure 2).

Plasmid pTP/Cla was used to generate the adenovirus vector Ad5(TPCla) by transfection of 293 cells essentially as described in Example 3.

Example 6: Effect of the intercistronic leader on the level of expression of DHFR

To establish the increased translational efficiency of $DHFR^r$ with the L1-3 in the intercistronic region upstream of the $DHFR_r$ coding sequence, the level of DHFR protein was measured in cells infected with Ad5-(CAT-DHFR) or Ad5(TPC1a) recombinant virus by Western transfer assay.

Sub-confluent 293 cells were infected with Ad5(CAT-DHFR) or Ad5(TPC1a) recombinant virus. The infected cells were incubated and harvested when the cells showed considerable cytopathic effects. The harvested cells were pelleted by centrifugation to remove the spent media. Excess media was removed by one rinse with phosphate buffered saline (PBS; Sigma, St. Louis, MO). The cells were resuspended in 0.25 M Tris HCl, pH 7.4. The cell suspensions were frozen and thawed three times to lyse the cells. Cell debris was removed by centrifugation and 50 ul of the supernatant was mixed with 50 ul 2x Sample Buffer (Table 1) and electrophoresed on a 15% acrylamide gel.

Table 1

2x Sample Buffer
36 ml 0.5 M Tris-HCl, pH 6.8
16 ml glycerol
16 ml 20% SDS
4 ml 0.5% Bromophenol Blue in 0.5 M Tris-HCl, pH 6.8

Mix all ingredients. Immediately before use, Add 100 ul Beta-mercaptoethanol to each 900 ul dye mix.

Western Buffer A

50 ml 1 M Tris-HCl, pH 7.4
20 ml 0.25 mM EDTA, pH 7.0
5 ml 10% NP-40
37.5 ml 4 M NaCl
2.5 g gelatin

Dilute the Tris, EDTA, NP-40 and NaCl to a final volume of 1 liter with distilled water. Add the gelatin to 300 ml of this solution and heat in the microwave oven until the gelatin has dissolved into solution. The gelatin solution is added back to the remainder of the first solution and stirred at 4°C until cool. The buffer is stored at 4°C.

The proteins were also transferred to a nitrocellulose filter using the method described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76:4350-4353, 1979). The nitrocellulose filter was immersed in Western Buffer A (Table 1) for 1 hour at room temperature. The buffer was removed and the filter was probed with an anti-DHFR antibody diluted in Western Buffer A for 1 hour at room temperature. The antibody solution was removed and excess antibody was removed from the filter by three washes with Western Buffer A. The protein bound by the antibody was visualized by a 1 hour, room temperature incubation with 125I -labelled Protein A diluted in Western Buffer A. The labelled Protein A solution was discarded and excess label was removed by three washes with Western Buffer A. The labelled filter was exposed to X-ray film for four hours at -80°C with an intensifying screen. The results show that $DHFR^r$ protein produced by cells infected with Ad5(TPCla) recombinant virus was present at high levels, but was not detected in cells infected with Ad5(CAT-DHFR) recombinant virus.

To confirm the production of polycistronic mRNA, BHK cells were transfected with pD5CAT-DHFR^r or a control plasmid containing a monocistronic DHFR expression unit. The mRNA produced by the transfectants was hybridized to a ^{32}P-labeled antisense DNA probe (Figure 3b) and the mixture was digested with S1 nuclease to remove the unhybridized probe. Analysis of the products (Figure 3a) indicated that pD5CAT-DHFR^r directed the expression of dicistronic mRNA (lanes 1 and 2, arrows), whereas the monocistronic plasmid directed the production of a smaller mRNA. The doublet observed with the dicistronic message is due to splicing in a portion of the transcripts as shown in Figure 3b. The top band in lanes 1 and 2 represents undigested probe DNA.

mRNA produced from cells transfected with a polycistronic expression unit containing an intercistronic tripartite leader was analyzed by Northern blot (Thomas, Proc. Natl. Acad. Sci. USA 77:5201, 1980). Results indicated that mRNA of the size predicted for the dicistronic message was produced.

Example 7: Construction of pTP/F9/Cla

A tricistronic transcription unit, comprising a factor IX cDNA, CAT gene and DHFR^r cDNA, was constructed from the factor IX cDNA and pBoel360 as shown in Figure 4. Plasmid pBoel360 (Example 3) was partially digested with Bam HI to linearize the plasmid. The adhesive ends were treated with calf alkaline phosphatase, essentially as described by Maniatis et al. (ibid.), to prevent self-ligation. A factor IX cDNA was isolated, as described by Kurachi and Davie (Proc. Natl. Acad. Sci. USA 79:6461-6464, 1982), as a 1.4 kb Bam HI fragment. The 1.4 kb Bam HI fragment was then subcloned into pUC13 to generate plasmid pF9pUC. Plasmid pF9pUC

was digested with Bam HI to isolate the 1.4 kb factor IX cDNA fragment which was joined with the linearized pBoel360 by ligation. The ligation mixture was transformed into E. coli strain HB101. Plasmid DNA was prepared and was screened by restriction enzyme digestion. A plasmid with the factor IX cDNA fragment inserted in the proper orientation and 5′ to the CAT cDNA sequence was designated pF9/Cla (Figure 4).

The tripartite leader sequence present in plasmid pTP(#323) was inserted between the translation termination codon of the factor IX cDNA and the translation initiation codon of the CAT cDNA as follows. Plasmid pF9/Cla was digested with Bam HI and Pst I to isolate the fragment comprising the 5′ coding sequence of the factor IX cDNA (1.4 kb). Plasmid pTP (#323) (Example 5) was linearized by partial digestion with Bam HI and treated with phosphatase. The linearized pTP was joined with the factor IX fragment by means of a Pst I Bam HI adapter.

The adapter, constructed from oligonucleotides ZC2029 (5′ GAT CTC ACC GTC TGC A 3′) and ZC2030 (5′ GAC GGT GA 3′) destroyed the Bam HI site but preserved the Pst I site on the factor IX fragment. The resultant plasmid was designated IX[TP] (#515). Plasmid pF9/Cla was digested with Ava I and Hind III, and the 4.9 kb fragment containing the pML-1 sequence was recovered. Plasmid pF9/Cla was also digested with Hind III and Ssp I, and the 1.5 kb fragment containing the MLP, L1-3 and 5′ factor IX sequences was recovered. The two pF9/Cla-derived fragments were then joined to the factor IX-L1-3 Ssp I-Ava I fragment from plasmid IX[TP]. The resultant plasmid was designated pTP/F9/Cla (Figure 4).

Plasmids pTP/F9/Cla and pF9/Cla were transfected into BHK cells and transient expression levels of chloramphenicol acetyl transferase were measured. CAT expression in pTP/F9/Cla transfectants

was approximately 20-fold higher than in pF9/Cla transfectants.

Example 8: Construction of pF7/TP/BIP

A. Construction of pX

Plasmid pDX was constructed from plasmid pD3′, a vector identical to pD3 except that the SV40 polyadenylation signal is in the late orientation. Thus pD3′ contains a Bam HI site as the site of gene insertion.

To generate pDX, the Eco RI site in pD3′ was converted to a Bcl I site by Eco RI cleavage, incubation with S1 nuclease, and subsequent ligation with Bcl I linkers. Plasmid DNA was prepared from a positively identified colony, and the 1.9 kb Xho I-Pst I fragment containing the altered restriction site was prepared via agarose gel electrophoresis. In a second modification, Bcl I-cleaved pD3 (Example 1) was ligated with kinased Eco RI-Bcl I adaptors (constructed from oligonucleotides ZC525, 5′ GGAATTCT 3′; and ZC526, 5′ GATCAGAATTCC 3′), in order to generate an Eco RI site as the position for inserting a coding sequence into the expression vector. Positive colonies were identified by restriction analysis, and plasmid DNA prepared from a positively identified colony was used to isolate a 2.3 kb Xho I-Pst I fragment containing the modified restriction site. The pD3 and pD3′ fragments were incubated together with T4 DNA ligase, transformed into E. coli HB101 and positive colonies were identified by restriction analysis. A plasmid containing the desired expression vector was designated pDX (Figure 5).

B. Construction of pFVII

An expression vector comprising the Ad5 ori, SV40 enhancer, Ad2 major late promoter and tripartite leader, 5′ and 3′ splice signals, a factor VII cDNA and

the SV40 polyadenylation signal is generated from plasmids pDX, pD11, and pFVII(565+2463)/pDX (ATCC Accession no. 40205).

As shown in Figure 5, the factor VII cDNA present in pFVII(565+2463)/pDX was modified to remove the 3' untranslated region. Plasmid pFVII(565+2463)/pDX was digested with Eco RI to isolate the 2.4 kb factor VII cDNA fragment. The Eco RI fragment was partially digested with Mbo II to isolate the 1.4 kb Eco RI-Mbo II fragment comprising the factor VII coding sequence. A synthetic oligonucleotide, designed to form an Mbo II-Bam HI adapter, was kinased and annealed. The 1.4 kb fragment was ligated with the kinased adapter and pUC13 which had been linearized by digestion with Eco RI and Bam HI. The resultant plasmid, pUCFVII, was digested with Eco RI and Bam HI to isolate the 1.4 kb fragment (fragment a).

Plasmid pDX is digested with Eco RI and Xba I to isolate the 4.0 kb fragment comprising the Ad5 ori, SV40 enhancer, Ad2 major late promoter and tripartite leader, 5' and 3' splice signals and pBR322 vector sequences (fragment b). Plasmid pD11 (Example 2) is digested with Bam HI and Xba I to isolate the 0.5 kb fragment comprising the SV40 polyadenylation signal and pML-1 vector sequences (fragment c). To construct pFVII fragments a, b, and c are joined in a three-part ligation.

C. Construction of plasmid pD11-BIP

An expression vector was constructed which comprised the Ad5 ori, the SV40 enhancer, the Ad2 major late promoter and tripartite leader, 5' and 3' splice signals, a BiP cDNA, and the SV40 polyadenylation signal was constructed from plasmids pD11 (Example 2), pBiP(pUC12) and pDX (Figure 5).

The immunoglobulin heavy chain binding protein (BiP) cDNA was obtained as a 2.4 kb Eco RI fragment in

pUC12 (described by Munro and Pelham, Cell 46:291-300, 1986) and was designated BiP(pUC12). The BiP cDNA was subcloned into the expression unit present in pD11 as follows. The BiP cDNA was isolated from the pUC12 vector sequences as two fragments. BiP(pUC12) was digested with Bam HI and Kpn I to isolate the 1.6 kb fragment and with Kpn I and Eco RI to isolate the 0.86 kb fragment. Plasmid pDX was digested with Eco RI and Xba I to isolate the 0.5 kb fragment comprising the SV40 polyadenylation signal and pML-1 vector sequences. Plasmid pD11 was digested with Bam HI and Xba I to isolate the 4.0 kb fragment comprising the Ad5 ori, SV40 enhancer, Ad2 major late promoter and tripartite leader and 5′ and 3′ splice signals and pML-1 vector sequences.

Plasmid pD11-BIP was generated by the ligation of the four fragments described above. This plasmid comprised the Ad5 ori, SV40 enhancer, Ad2 major late promoter and tripartite leader and 5′ and 3′ splice signals, BiP cDNA, SV40 polyadenylation signal and pML-1 vector sequences.

D. Construction of Plasmid pFVII-TP-BIP

A dicistronic transcription unit containing the factor VII and BiP cDNAs is constructed from plasmids pFVII, pD11-BIP and pTP as shown in Figure 5.

Plasmid pD11-BIP is digested with Eco RI and Eco RV to isolate the .5 kb fragment comprising the 5′ portion of the BiP cDNA. The .5 kb fragment is joined with an Eco RV-Xba I synthetic adapter and pUC13 which has been linearized by digestion with Eco RI and Xba I. The resultant plasmid is linearized by digestion with Nae I which cleaves the BiP fragment 5 bp 5′ to the translation initiation codon. The linearized plasmid is recircularized by ligation with synthetic Bam HI linkers. The resultant plasmid is linearized by digestion with Bam HI and joined by ligation with the 0.2 kb Bam HI fragment, comprising the tripartite

leader, obtained from Bam HI digested pTP (Example 5). The resultant plasmid is partially digested with Bam HI and completely digested with Eco RV to isolate the 0.7 kb fragment comprising the tripartite leader and 5′ BiP coding sequence.

The remaining fragments are obtained as follows. Plasmid pFVII is digested with Hind III and Bam HI to isolate the 2.1 kb fragment comprising the Ad2 major late promoter and tripartite leader, 5′ and 3′ splice signals and the factor VII cDNA. Plasmid pD11-BIP is digested with Hind III and Eco RV to isolate the 5.5 kb fragment comprising the SV40 enhancer, Ad5 ori, pML-1 vector sequences, SV40 polyadenylation signal and the 3′ 1.9 kb of the Bip cDNA.

The three fragments (0.7 kb tripartite leader and 5′ BiP, 2.1 kb pFVII, and 5.5 kb pD11-BIP) are joined in a three-part ligation. The resultant plasmid is designated pFVII-TP-BIP.

Claims

1. A polycistronic transcription unit which provides for enhanced expression of at least one cistron contained therein, of the formula:

$P\text{-}C_1\text{-}(HEL\text{-}C_n)_m$, wherein

P is a transcriptional promoter;

C is a DNA sequence encoding a protein;

HEL is a high efficiency leader;

n is a positive integer greater than zero; and

m is an integer from 1 to 8, inclusive.

2. The polycistronic transcription unit of claim 1 further including a leader (L) positioned downstream of the promoter (P) and upstream of C_1.

3. The polycistronic transcription unit of claim 2 wherein said leader is selected from the group consisting of the SV40 leader, adenovirus first leader, adenovirus tripartite leader, adenovirus L1-IX leader and ovalbumin leader.

4. The polycistronic transcription unit of claim 2 wherein the leader is a viral leader.

5. The polycistronic transcription unit of claim 4 wherein said viral leader is selected from the group consisting of adenovirus first leader, adenovirus tripartite leader, adenovirus L1-IX leader and SV40 leader.

6. The polycistronic transcription unit of claim 1 wherein said high efficiency leader is a high efficiency viral leader.

7. The polycistronic transcription unit of claim 1 wherein C_1 and C_n are subunits of a multi-subunit protein.

8. The polycistronic transcription unit of claim 7 wherein said multi-subunit protein is selected from the group consisting of factor XIII, platelet derived growth factor, immunoglobulins and histocompatibility antigens.

9. The polycistronic transcription unit of claim 1 wherein C_1 encodes a protein selected from the group consisting of protein C, factor VII, factor IX, factor X and factor VIII.

10. The polycistronic transcription unit of claim 1 wherein C_n encodes a protein selected from the group consisting of BiP, the S. cerevisiae KEX2 gene product, protein S, and von Willebrand factor.

11. The polycistronic transcription unit of claim 1 wherein C_1 encodes protein C and C_n encodes a protein selected from the group consisting of protein S and the S. cerevisiae KEX2 gene product.

12. The polycistronic transcription unit of claim 1 wherein C_1 encodes factor VIII and C_n encodes von Willebrand factor.

13. A cultured cell derived from a multicellular organism into which has been introduced a polycistronic transcription unit according to any of claims 1-12.

14. The cultured cell of claim 13 wherein said cultured cell is a mammalian cell.

15. A method for enhancing the expression of proteins in cultured cells derived from a multicellular organism comprising:

introducing into a cultured host cell derived from a multicellular organism a polycistronic transcription unit according to any of claims 1-12; and growing said cultured host cell in an appropriate medium.

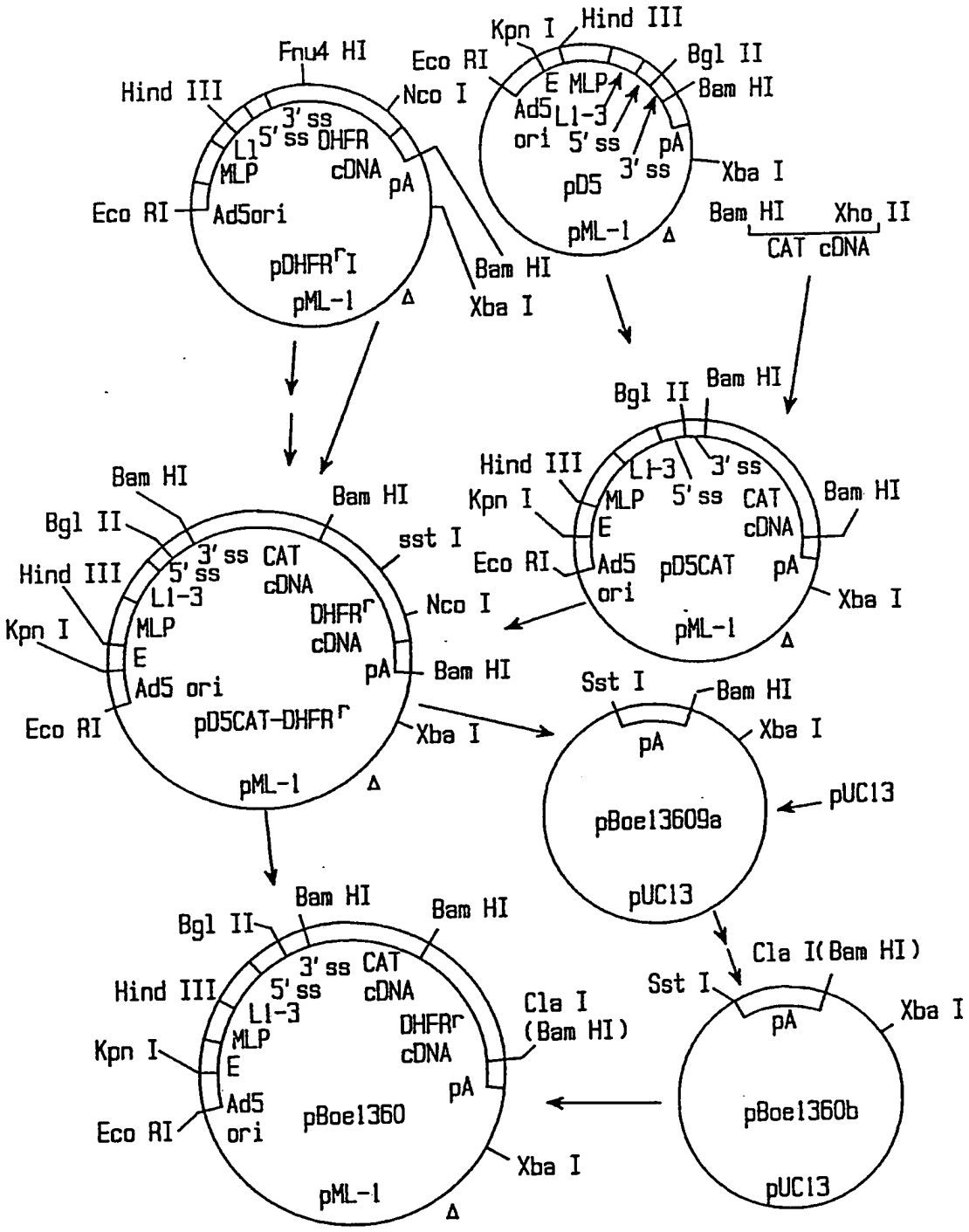

Fnu4 HI
Hind III
Nco I
3' ss
5' ss
DHFR
L1
MLP
cDNA
pA
Eco RI
Ad5ori
pDHFR
pML-1
Bam HI
Xba I
Kpn I
Hind III
Eco RI
Bgl II
E MLP
Ad5
ori
L1-3
5' ss
3' ss
pA
Xba I
pD5
pML-1
Bam HI
Xho II
CAT cDNA
Bgl II
Bam HI
Hind III
Kpn I
L1-3
3' ss
MLP
5' ss
CAT
cDNA
E
Ad5
ori
pD5CAT
pA
Xba I
pML-1
Bam HI
Bgl II
Hind III
Kpn I
Eco RI
3' ss
5' ss
L1-3
MLP
E
Ad5 ori
CAT
cDNA
DHFR
cDNA
pA
sst I
Nco I
Bam HI
pD5CAT-DHFR
Xba I
pML-1
Sst I
Bam HI
pA
Xba I
pUC13
pBoe13609a
pUC13
Cla I(Bam HI)
Sst I
pA
Xba I
pBoe1360b
pUC13
Bam HI
Bgl II
Bam HI
Hind III
Kpn I
Eco RI
3' ss
5' ss
L1-3
MLP
E
Ad5
ori
CAT
cDNA
DHFR
cDNA
pA
Cla I
(Bam HI)
pBoe1360
Xba I
pML-1

FIG.1

FIG.2

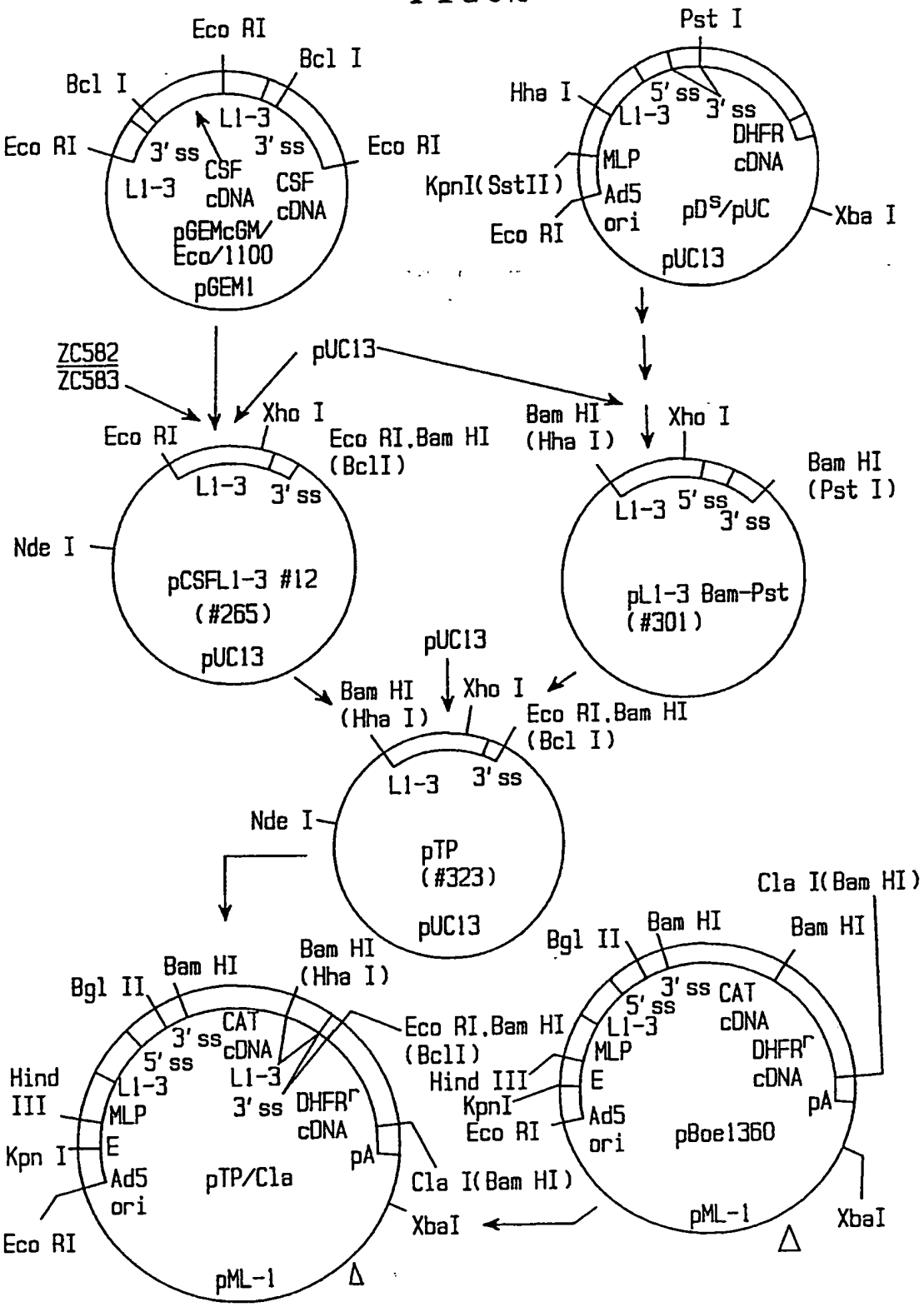

Eco RI
Bcl I
Bcl I
Eco RI
L1-3
3' ss
3' ss
Eco RI
L1-3
CSF cDNA
CSF cDNA
pGEMcGM/ Eco/1100
pGEM1
Pst I
Hha I
5' ss
L1-3
3' ss
DHFR cDNA
MLP
KpnI(SstII)
Ad5 ori
pDs/pUC
Eco RI
Xba I
pUC13
ZC582
ZC583
pUC13
Xho I
Eco RI
Eco RI.Bam HI (BclI)
L1-3
3' ss
Nde I
pCSFL1-3 #12 (#265)
pUC13
Bam HI (Hha I)
Xho I
Bam HI (Pst I)
L1-3
5' ss
3' ss
pL1-3 Bam-Pst (#301)
pUC13
Bam HI (Hha I)
Xho I
Eco RI.Bam HI (Bcl I)
L1-3
3' ss
Nde I
pTP (#323)
pUC13
Cla I(Bam HI)
Bam HI
Bam HI
Bgl II
3' ss
5' ss
CAT cDNA
L1-3
MLP
E
Ad5 ori
DHFR cDNA
pA
pBoe1360
pML-1
XbaI
Bam HI
Bam HI (Hha I)
Bgl II
CAT cDNA
3' ss
5' ss
L1-3
3' ss
DHFR cDNA
Eco RI.Bam HI (BclI)
Hind III
MLP
E
Kpn I
Ad5 ori
pA
pTP/Cla
Cla I(Bam HI)
Eco RI
XbaI
pML-1

FIG. 3A

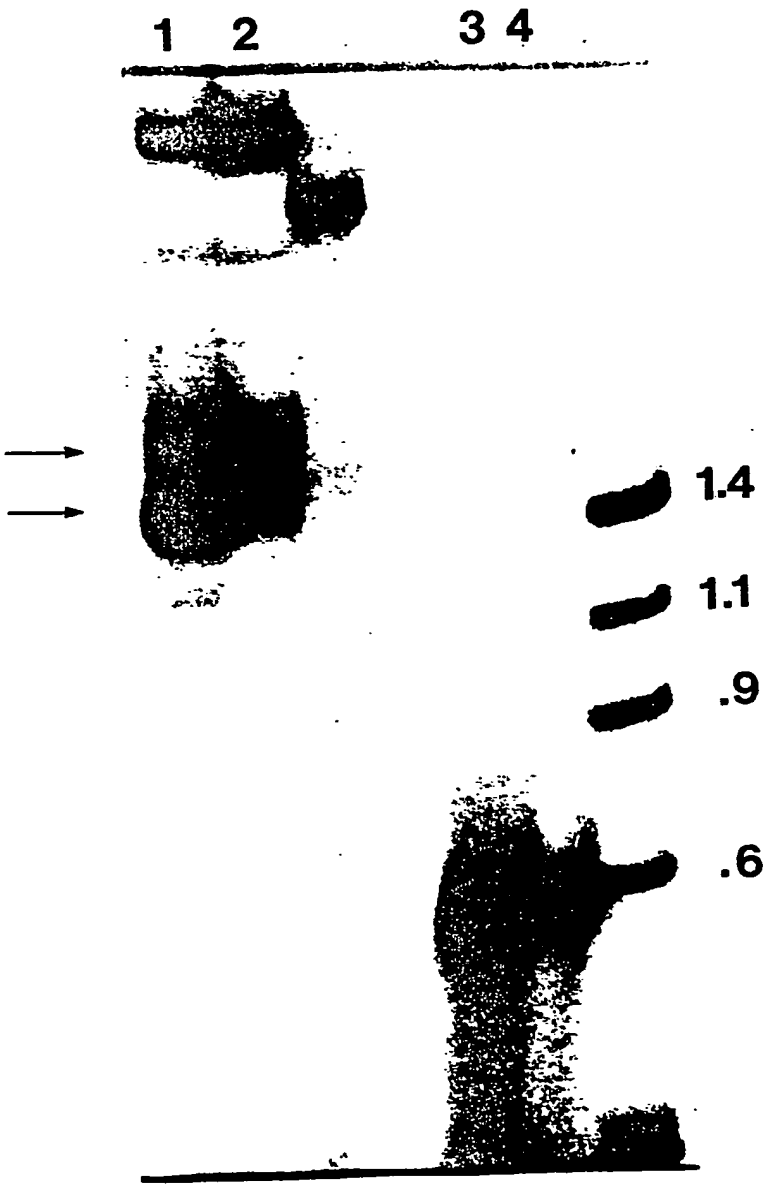
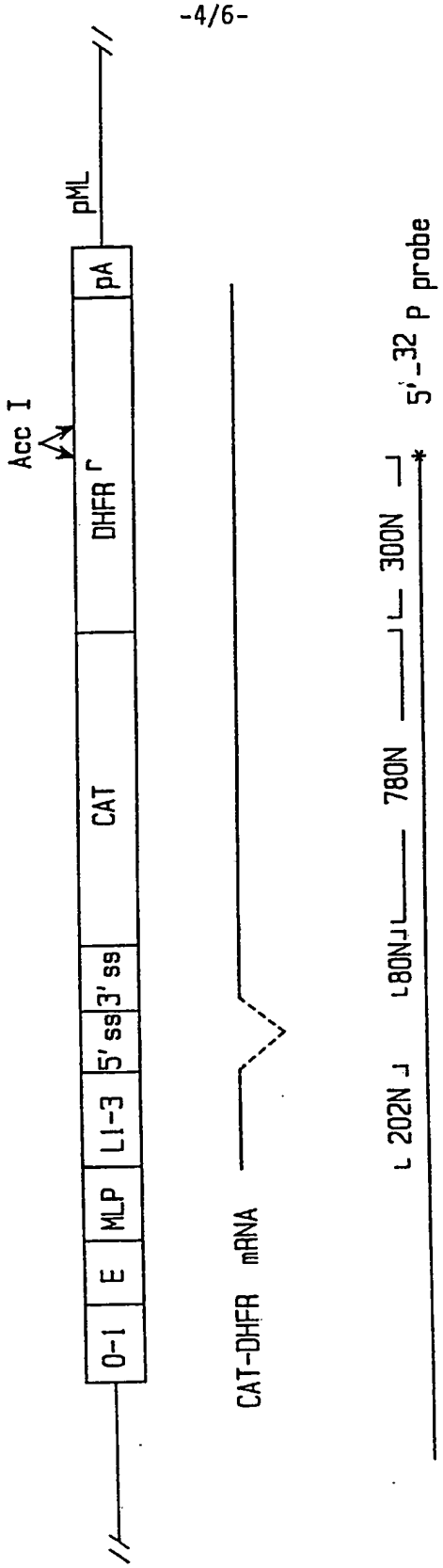


FIG.3B


pML
pA
DHFR
Acc I
CAT
3' ss
5' ss
L1-3
MLP
E
0-1
CAT-DHFR mRNA
202N
180N
780N
300N
5'-32 P probe

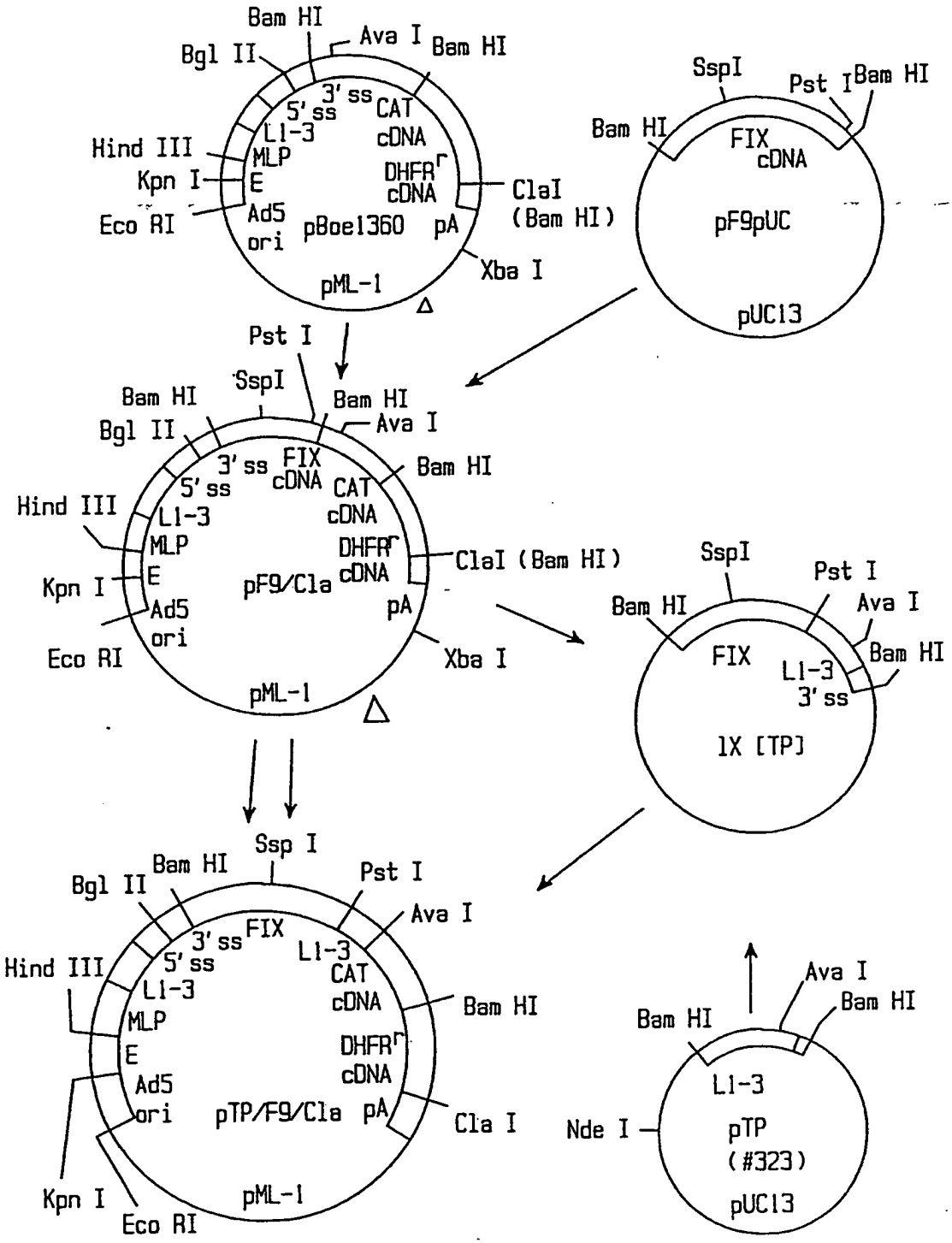
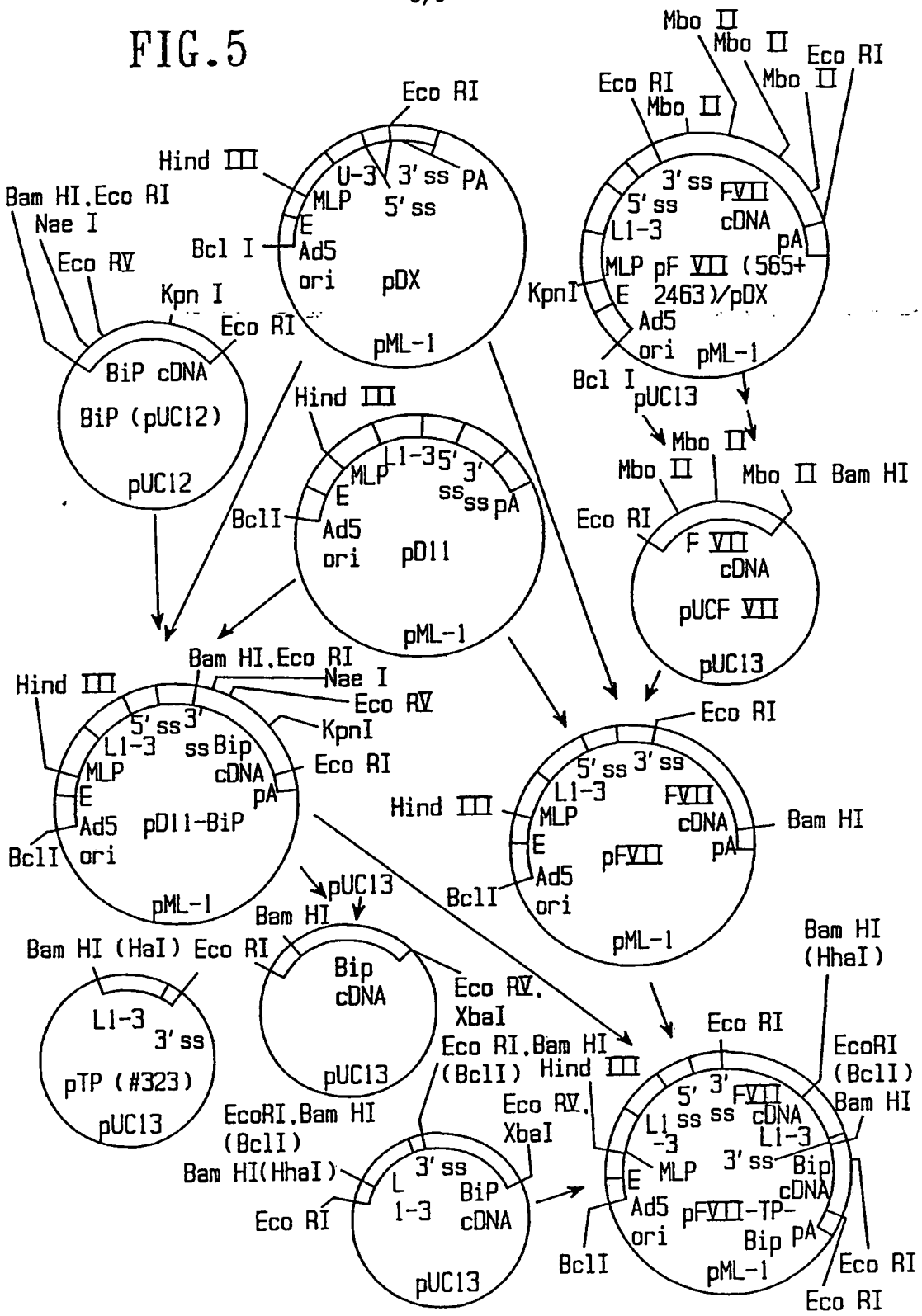

Bam HI
Ava I
Bam HI
Bgl II
3' ss
5' ss
CAT
L1-3
cDNA
Hind III
MLP
DHFR
Kpn I
E
cDNA
ClaI
(Bam HI)
Ad5
Eco RI
ori
pBoe1360
pA
Xba I
pML-1
SspI
Pst I
Bam HI
Bam HI
FIX
cDNA
pF9pUC
pUC13
Pst I
SspI
Bam HI
Bam HI
Ava I
Bgl II
3' ss
FIX
cDNA
5' ss
CAT
Bam HI
Hind III
L1-3
cDNA
MLP
DHFR
ClaI (Bam HI)
Kpn I
E
pF9/Cla
cDNA
pA
Ad5
ori
Xba I
Eco RI
pML-1
SspI
Pst I
Bam HI
Ava I
FIX
L1-3
Bam HI
3' ss
1X [TP]
Ssp I
Bam HI
Pst I
Bgl II
Ava I
FIX
3' ss
L1-3
Hind III
5' ss
CAT
L1-3
cDNA
Bam HI
MLP
DHFR
E
cDNA
Ad5
ori
pTP/F9/Cla
pA
Cla I
Kpn I
pML-1
Eco RI
Ava I
Bam HI
Bam HI
L1-3
Nde I
pTP
(#323)
pUC13

FIG.4

FIG.5


Eco RI
Hind III
Bam HI.Eco RI
Nae I
Eco RV
Kpn I
Eco RI
BiP cDNA
BiP (pUC12)
pUC12
U-3
3' ss
PA
MLP
5' ss
E
Bcl I
Ad5
ori
pDX
pML-1
Mbo II
Mbo II
Eco RI
Eco RI
Mbo II
Mbo II
3' ss
5' ss
FVII
L1-3
cDNA
pA
MLP pF VII (565+
KpnI
E 2463)/pDX
Ad5
ori
pML-1
Bcl I
pUC13
Mbo II
Mbo II
Mbo II Bam HI
Eco RI
F VII
cDNA
pUCF VII
pUC13
Hind III
L1-3
5' ss
3' ss
MLP
E
pA
BclI
Ad5
ori
pD11
pML-1
Hind III
Bam HI.Eco RI
Nae I
Eco RV
KpnI
Eco RI
5' ss
3' ss
L1-3
Bip
cDNA
pA
MLP
E
Ad5
pD11-BiP
BclI
ori
pML-1
Eco RI
5' ss
3' ss
L1-3
FVII
Hind III
MLP
cDNA
Bam HI
E
pA
pFVII
BclI
Ad5
ori
pML-1
pUC13
Bam HI
Bam HI (HaI) Eco RI
Bip
cDNA
Eco RV.
XbaI
L1-3
3' ss
pTP (#323)
pUC13
pUC13
Bam HI
(HhaI)
Eco RI
Eco RI.Bam HI
(BclI) Hind III
EcoRI
(BclI)
Eco RV.
XbaI
5' ss
3' ss
FVII
Bam HI
EcoRI.Bam HI
(BclI)
L1 ss ss cDNA
-3
L1-3
Bam HI(HhaI)
3' ss
Bip
cDNA
E
MLP
Eco RI
L
1-3
BiP
cDNA
Ad5
ori
pFVII-TP-
Bip pA
BclI
pML-1
Eco RI
pUC13
Eco RI

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03228

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/67, C 12 N 15/85, C 12 N 15/10

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	WO, A, 88/05466 (CODON) 28 July 1988, see page 4, line 1 - page 7, line 27; claims	1
X	EP, A, 0117058 (GENENTECH) 29 August 1984, see the whole document	1
X	EP, A, 0154576 (INTERFERON SCIENCES, INC.) 11 September 1985, see claims	1
A	EP, A, 0219214 (K.K. YAKULT HONSHA) 22 April 1987	
X	Chemical Abstracts, vol. 107, no. 21, 23 November 1987, (Columbus, Ohio, US), V.V. Kravchenko et al.: "Construction and properties of artificial polycistrons containing truncated E. coli tryptophan operon gene and M13 coat protein gene", see page 206, abstract 192226a & Bioorg. Khim. 1987, 13(9), 1176-85	1

./.

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th October 1989	12. 12. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, vol. 108, no. 5, 1 February 1988, (Columbus, Ohio, US), see page 504, abstract 36330v & JP, A, 6291199 (FUJISAWA PHARMACEUTICAL CO. LTD) 25 April 1987 --	
X	WO, A, 87/02707 (GENETICS INSTITUTE, INC.) 7 May 1987, see page 12, line 12 - page 19, line 5 --	1
X	Chemical Abstracts, vol. 107, no. 11, 14 September 1987, (Columbus, Ohio, US), E. Boel et al.: "Expression of human pancreatic polypeptide precursors from a dicistronic mRNA in mammalian cells", see page 206, abstract 91240v & FEBS Lett. 1987, 219(1), 181-8 (cited in the application) --	1
A	Gene, vol. 34, 1985, Elsevier Science Publishers S.Johnston et al.: "High-level expression of M13 gene II protein from an inducible polycistronic messenger DNA", pages 137-145 --	
P,A	Chemical Abstracts, vol. 109, no. 21, 21 November 1988,(Columbus, Ohio, US), K.L. Berkner: "Development of adenovirus vectors for the expression of heterologous genes", see page 155, abstract 184334k & BioTechniques 1988, 6(7), 616-18, 620-4, 626,628-9 --	
A	Nucleic Acids Research, vol. 13, no. 3, 1985 K.L. Berkner et al.: "Effect of the tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant", pages 841-857 (cited in the application) --	
A	Proc. Natl. Acad. Sci, USA, vol. 82, February 1985 (US) R.J. Kaufman: "Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors" pages 689-693 (cited in the application) ----------	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8903228 SA 30255

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 04/12/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8805466	28-07-88	AU-A- 1187588	10-08-88
EP-A- 0117058	29-08-84	US-A- 4713339	15-12-87
		AU-B- 578011	13-10-88
		AU-A- 2353184	26-07-84
		JP-A- 59173096	29-09-84
EP-A- 0154576	11-09-85	JP-A- 60199387	08-10-85
EP-A- 0219214	22-04-87	JP-A- 62051991	06-03-87
WO-A- 8702707	07-05-87	EP-A- 0247145	02-12-87